

NUTRIENT REQUIREMENTS AND OTHER FACTORS INVOLVED IN THE CULTURE OF HUMAN KIDNEY CELLS ON MICROCARRIER BEADS

Marian L. Lewis, Ph.D.
Technology Inc.
Houston, Texas

Dennis R. Morrison, Ph.D.
NASA - Johnson Space Center/SD4
Houston, Texas

This report describes the culture of human kidney cells on microcarrier beads in the Bioprocessing Laboratory at NASA/Johnson Space Center. These were the first series of studies done before and during 1983 to determine optimum culture conditions including medium type and bead type and density. The composition of several medium types and the molecular weights of some common culture medium supplements and cellular proteins are included for information. The report also describes the microgravity cell-to-bead attachment experiment performed on STS-8.

CELLS AND BEADS

The human kidney (HK) cells used in these studies were obtained from a commercial vendor (M. A. Bioproducts) as monodisperse frozen primary cells. The cultured cells were characterized in our laboratory as normal human diploid, anchorage dependent cells with a finite lifespan of 8.3 population doublings. This cell type was chosen because of production of high levels of urokinase (UK), heterogeneity of electrophoretically separable subpopulations and relative ease of culture. Four morphological subtypes were identified microscopically as fibroblastoid, fenestrated, and large and small epithelioid cells. The diameter size range is approximately 10 to 30 microns and the generation time in culture is 24 hours.

The microcarrier beads, Cytodex 3, used in these studies were obtained from Pharmacia Fine Chemicals and consisted of a thin layer of denatured collagen chemically bonded to a cross-linked dextran matrix.² Table 8-1 shows characteristics of these microcarriers.

SELECTION OF CULTURE MEDIUM

Several types of commercially available media were tested for their ability to support cell growth in our laboratory. These media included M-199, Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium Alpha (MEM- α), and a medium developed in our laboratory, Microcarrier Medium (MM), which

consisted of the above listed media plus other additives. Components of Microcarrier Medium are shown in table 8-2. Before mixing with cells, the media were supplemented with 10% fetal calf serum, 100 units and 100ug per ml of penicillin and streptomycin.

Figure 8-1 shows results of a study comparing media to determine which type produced the best outgrowth. The cells were seeded at low density of about 50,000/ml. Counts showed a two-day attachment and lag period followed by log growth to day 7. In this test the medium was not changed to replace depleted nutrients and the cells were degenerating by day 10 before reaching a high density. Based on this and repeated tests showing the same result, we selected MM as the medium of choice for cell growth. Although DMEM did not support outgrowth as well as MM, the cells appeared to survive longer. This medium would be a candidate in cases which slower growing cells would be desirable.

COMPARISON OF MICROCARRIER TYPES

A series of studies were done to define cell growth on several different types of microcarriers. Figure 8-2 shows results of one such study. Biosilon beads (Nunc) are polystyrene, Cytospheres (Lab-Tek Division, Miles Laboratories) are non-toxic plastic surface treated beads, and Cytodex 1, 2, and 3 are cross-linked dextran matrix (Pharmacia Fine Chemicals). Cytodex 1 beads bear charged DEAE groups throughout the bead and Cytodex 2 beads have a surface layer of charged DEAE. The Cytodex 3 beads have a collagen layer bonded to the surface of the beads.

Figure 8-2 shows that cells seeded in plates with the Cytodex 3 beads attached and grew much better than those seeded on Cytodex 1, 2, or Cytospheres. Biosilon beads appeared to be a second choice if it should become desirable to culture cells on polystyrene beads. No follow-up work has been done to define cell survival for longer than 7 days on the Biosilon beads.

EFFECT OF BEAD CONCENTRATION ON CELL SURVIVAL

To determine the survival of cells in a no-headspace system at different concentrations, cultures were set up using passage 3 HK cells. When the cells were about 90% confluent on the beads, the beads were divided into suspension sets containing 10, 20, and 30 mg/ml. These suspensions were then transferred to syringes and placed in the incubator. As shown in figure 8-3, the survival of cells in syringes with beads at 10 mg/ml was superior to the higher concentrations. This was expected since oxygen depletion would occur more rapidly at higher cell concentrations. The time at which approximately half of the cells were no longer on the beads for each concentration was two

days for beads at 30 mg/ml, 2.75 days for beads at 20 mg/ml, and 4 days for beads at 10 mg/ml. These studies were preliminary to the more sophisticated experiments designed later to define the oxygen usage of the HK cells.

NORMAL CULTURE CONDITIONS DEVELOPED FROM THESE STUDIES

Based on these studies, the cells are normally grown on the Cytodex 3 beads in static petri plate cultures. The initial seeding density for beads is 5 mg/ml and cells are set up at 50,000/ml. The total volume per dish is 20 ml. Cells are usually confluent in 7-10 days and the cell yield is about 300,000 to 500,000 cells/ml depending on passage level and general condition of the cells when they are set up.

ATTACHMENT OF CELLS TO BEADS IN MICROGRAVITY

Having defined the optimal parameters of medium type and bead type and density for growth of the KH cells in our laboratory, we wanted to determine the behavior of the cells in microgravity. In 1983, we developed an experiment to determine if cells will attach to beads in space. Since these anchorage dependent cells will not survive in a suspended state, it is important to determine if they will attach to substrata in the microgravity environment. Potential applications of this information include providing growth surfaces to cells separated by continuous flow electrophoresis and directing seeding cultures in the bioreactor while on-orbit.

On STS-8, cells and beads were mixed on-orbit in incubated growth chambers.^{3,4} At selected time intervals, glutaraldehyde was injected into the chambers and after the flight, cell attachment was evaluated. Counts of beads with cells attached showed that the cells attached to beads as well and possibly better in microgravity than in the ground-based control experiment. Figure 8-4 shows a scanning electron micrograph of a cell attached to a bead surface from a ground control sample fixed 5 minutes after mixing cells and beads. After 24.5 hours post mixing the beads with cells, some bead surfaces were almost confluent in both the flight and ground experiment as shown in figure 8-5. There were no apparent differences in the manner in which the cells were attached between the flight and ground control. This preliminary experiment showing that it is possible to seed cultures in space is a precursor for future microgravity bioreactor experiments.

CULTURE MEDIUM CONSIDERATIONS FOR THE BIOREACTOR

Nutrient depletion is a primary consideration in the mass culture of cells. In order to replenish those components which are rapidly utilized by the

cells, it is desirable to define the amounts in culture medium before and during cell culture. This may be done for the amino acids by HPLC. Figure 8-6 shows a typical amino acid profile. The figure translates into the actual concentrations shown in table 8-3 for a cell culture maintained on serum free medium for 16 days. As the bioreactor project develops, experiments will be done to define a plan for resupplying depleted amino acids and other nutrients. In cases in which it is desirable to grow the cells in the bioreactor instead of just maintaining them, additional growth supplements such as fetal calf serum must be added to the medium. With addition of serum, the medium is no longer defined, thus evaluating nutrient depletion becomes complicated. One way to reduce the ambiguities due to serum is to use defined serum such as than obtainable from HyClone.

In addition to the components listed for the HyClone serum there are other supplements which are sometimes found in cell culture media. Some of these are listed with their molecular weights in table 8-4. Table 8-5 shows the molecular weights of some proteins found in cells. The high molecular weight proteins must be considered in the product concentration loop design since several are in the same range as the plasminogen activators and other natural cell products which have been suggested for concentration and harvest.

CONCLUSIONS

Human kidney cells have been successfully cultured on microcarrier beads in a growth medium developed in our laboratory. These studies were done using light seeding densities of approximately 2-3 cells per bead and the cells were grown in static petri plate cultures. Seeding cultures at 50,000 cells/ml and a bead density of 5 mg/ml resulted in a lag period of about 2 days before beginning of the log growth phase. Cultures reached confluence in 7 to 10 days.

Having defined the optimum culture conditions in ground based studies, cell attachment experiments were flown on STS-8 which showed that cell attachment in microgravity equaled and was perhaps superior to that on Earth. These studies are preliminary to development of the cell biology experiments being designed for the flight bioreactor.

REFERENCES

1. Lewis, Marian L., Morrison, Dennis R., Mieszkic, Bernard J., and Fessler, Diane L., "Problems in the Bioassay of Products from Cultured HEK Cells: Plasminogen Activator," In Eukaryotic Cell Cultures, Eds., R. T. Action and J. D. Lynn, Plenum Publishing Corp., pp. 241-267, 1984.

2. Microcarrier Cell Culture Principles and Methods, Pharmacia Fine Chemicals, Almquist and Wiksell Trykeri AB, Sppsala, Sweden, pp. 64, December 1981.
3. Lewis, Marian L., Cogoli, Augusto, Morrison, Dennis R., and Tschopp, Alexander, "Anchorage Dependent Cells Attach to Microcarrier Beads in Microgravity," Eighth International Biophysics Congress, Bristol, U.K., 1984.
4. Tschopp, Alexander, Cogoli, Augusto, Lewis, Marian L., and Morrison, Dennis R., "Bioprocessing in Space: Human Cells Attached to Beads in Microgravity," Jour. Biotechnology, 1:287-293, 1984.

TABLE 8-1.- CHARACTERISTICS OF MICROCARRIERS

<u>Type</u>	<u>Cytodex 3 (Pharmacia)</u>
Density (in 0.9% NaCl)	1.04 G/ML
Size (mean diameter)	175 microns
Size (range)	133-215 microns
Approximate area	4,600 cm ² /g dry weight
Approximate number beads/g dry weight	4.0 × 10 ⁶
Swelling factor (in 0.9% NaCl)	14 ml/g dry weight

Cytodex 3 beads consist of a thin layer of denatured collagen chemically coupled to a matrix of cross-linked dextran.

TABLE 8-2.- MICROCARRIER MEDIUM

<u>Component</u>	<u>MFR</u>	<u>Weight or Volume</u>
M-199	Gibco	1 pkt
Mem-Alpha	Gibco	1 pkt
DME (w/4.5 g glucose)	Gibco	1 pkt
NaHCO ₃	Sigma	16.2 g
Bactopeptone	Difco	1.2 g
Folic Acid	Sigma	0.02 g
i-Inositol	Sigma	0.072 g
Nicotinic Acid 0.5 mg/ml	Sigma	2 ml
Deionized High Purity H ₂ O	-	2776 ml
Fetal Bovine Serum	-	300 ml
Penicillin/Streptomycin	-	100 units/100ug/ml Final concentration
Hepes	-	10 mM Final concentration

TABLE 8-3.- AN EXAMPLE OF AMINO ACIDS IN SERUM-FREE MEDIUM
CONDITIONED BY HEK CELLS

Sample: T25616
I.D. Run Number: 18-181
Date: July 1, 1985

<u>Amino Acid</u>	<u>Amount Applied</u> <u>(Nanomole)</u>	<u>Concentration</u> <u>(Micromole/ml)</u>
Carboxymethyl-cys	1.229	0.54
ASP	1.095	0.48
THR	2.842	1.25
GLN	*	0.00
SER	2.339	1.03
GLU	3.366	1.48
PRO	1.306	0.57
GLY	289.13	127.22
ALA	1.371	0.60
CYS	0	0.00
VAL	2.756	1.21
MET	0	0.00
ILE	2.158	0.95
LEU	4.971	2.19
TYR	.962	0.42
PHE	1.54	0.68
HIS	.507	0.22
LYS	3.924	1.73
ARG	1.176	0.52

*GLN is not resolved. It co-elutes with THR.

TABLE 8-4.- MOLECULAR WEIGHTS
Some Supplements to Cell Culture Medium

<u>Component</u>	<u>MW</u>
Human fibronectin	~220,000
Transferrin	~85,000
Insulin (bovine)	5,700
Selenous acid (Na_2SeO_3 or NA selenite)	
Ascorbic acid	176.1
Glutamine	146.1
Glucose	~180
BSA	70,000
Ethanolamine	~142
Lipids (Phospho-)	750
Alanine	89
Lactic acid	~101
Ficol 400	400,000
Epidermal growth factor	6,100
Dextran T-70	~70,000
Dextran T-500	~500,000
Fibronectin	~220,000
Poly-D-Lysine	~70,000

TABLE 8-5.- MW'S OF SOME CELLULAR PROTEINS (5,000 1×10^6 RANGE)

Some Cellular Enzymes

Tryptophan synthetase		159,000
Aspartate transcarbamylase (<u>E. Coli</u>)		310,000
Glutamine synthetase (<u>E. Coli</u>)		592,000
Pyruvate dehydrogenase complex		7×10^6
Polypeptides	100 - 300 AA's	12,000-36,000

Some Serum Components

Serum albumin	550 AA's	68,000
α_1 -Globulins		40,000-55,000
α_1 -Lipoproteins		200,000-400,000
α_2 -Globulins		Up to 800,000
β_1 -Lipoproteins		$3-20 \times 10^6$
γ Globulins		150,000
Fibrinogen		340,000
Plasminogen		90,000

Other

Myoglobin (prob. not in our cells)		16,900
Myosin		470,000

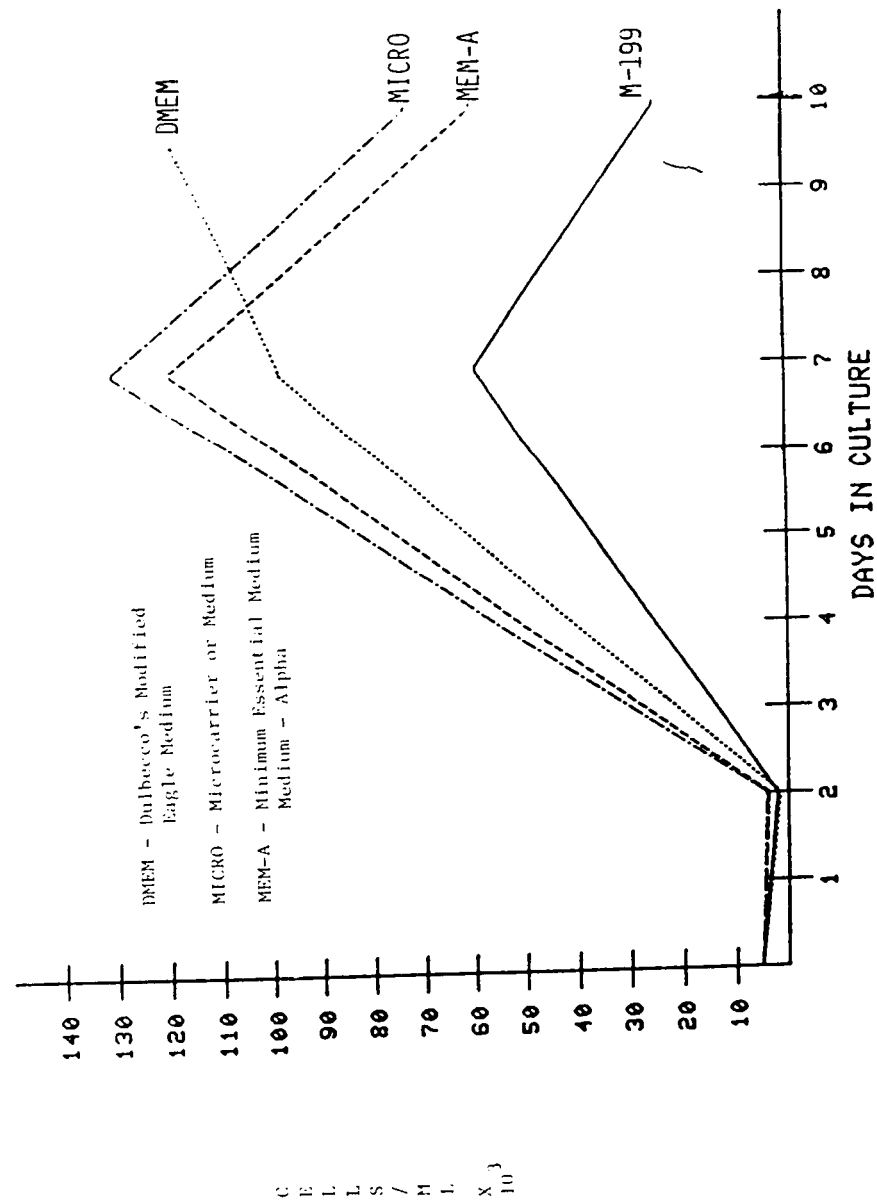


Figure 8-1.- Comparison of medium types for outgrowth of cells on Cytodex 3 microcarriers.

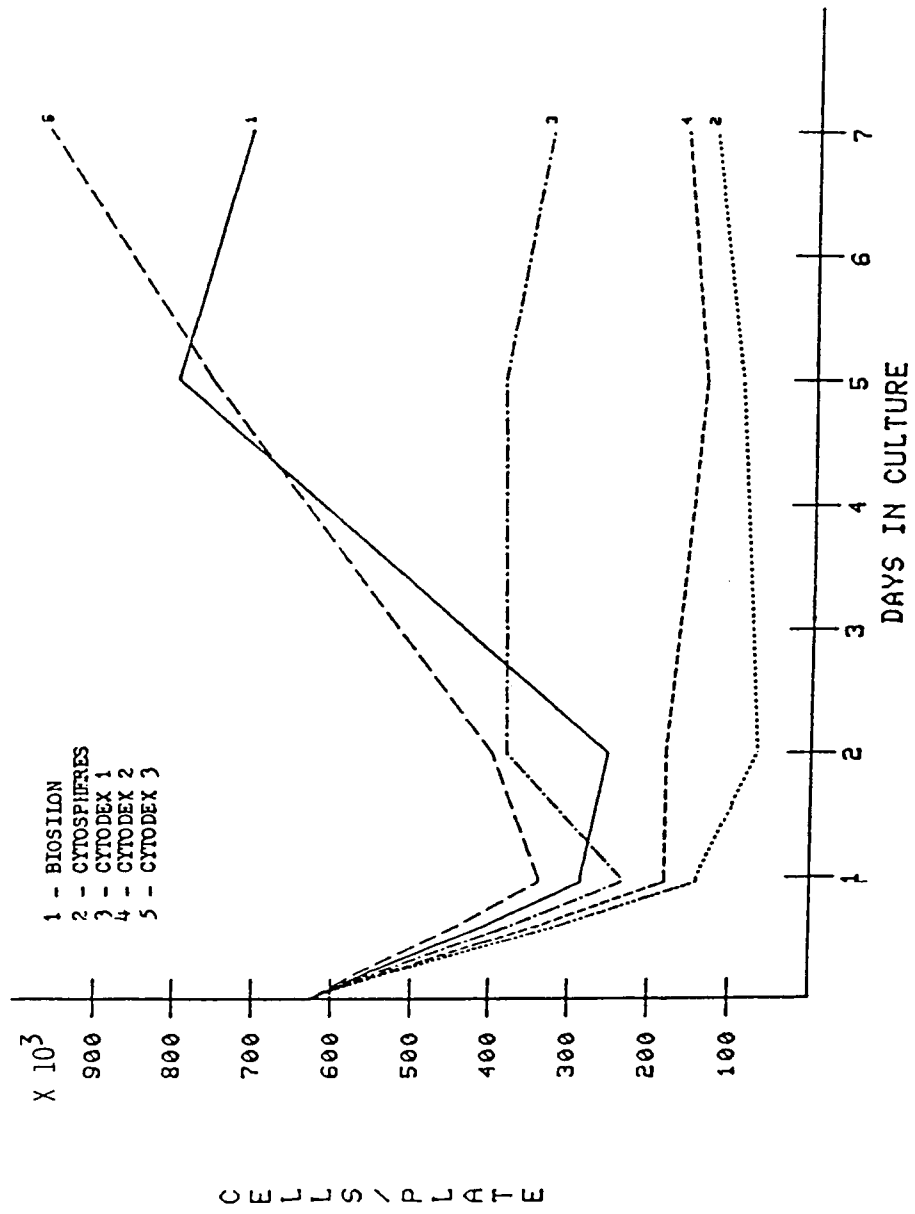


Figure 8-2.- Microcarrier comparison study.

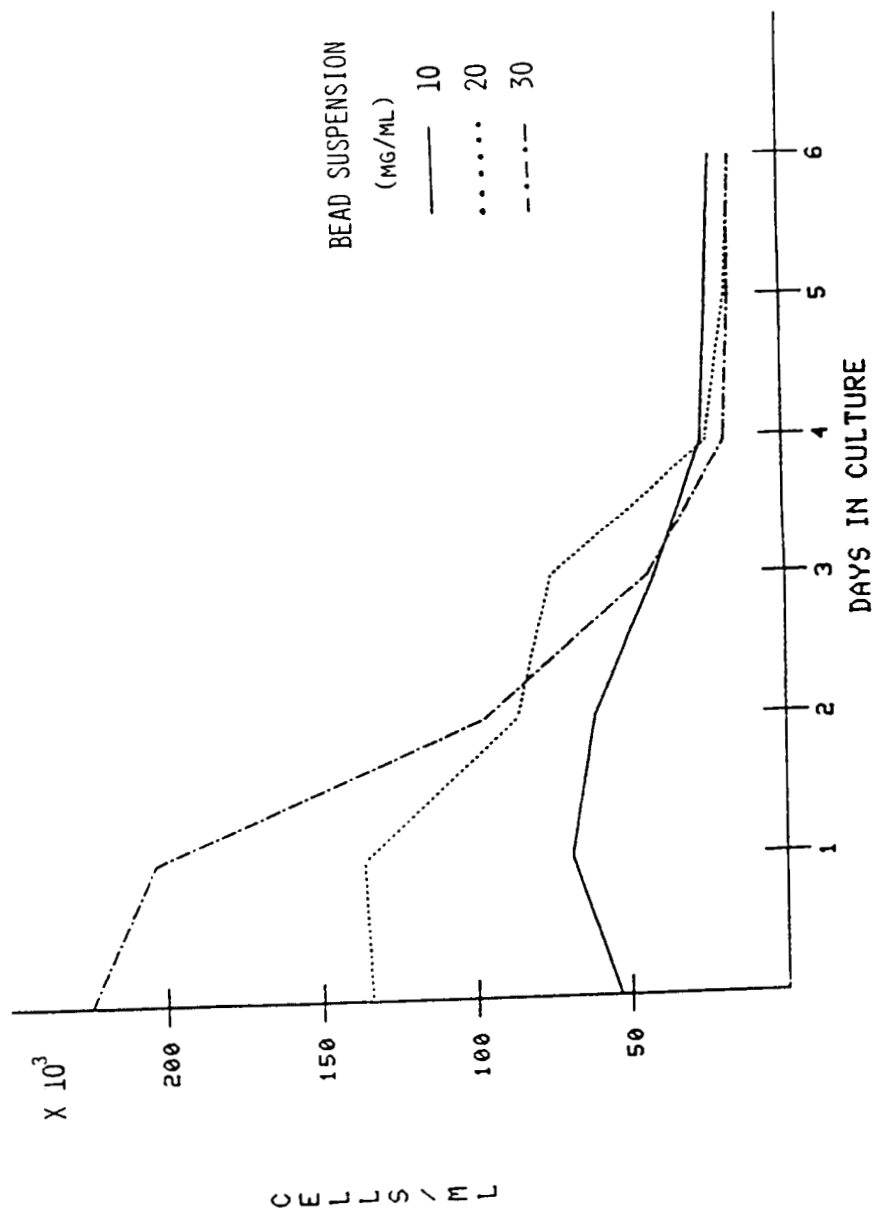


Figure 8-3.- Microcarrier syringe cultures
HEK 4347 p-3 on Cytodex 3-90% confluent.

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Figure 8-4.- Ground control-fixed 5 min. after mixing cells and beads.

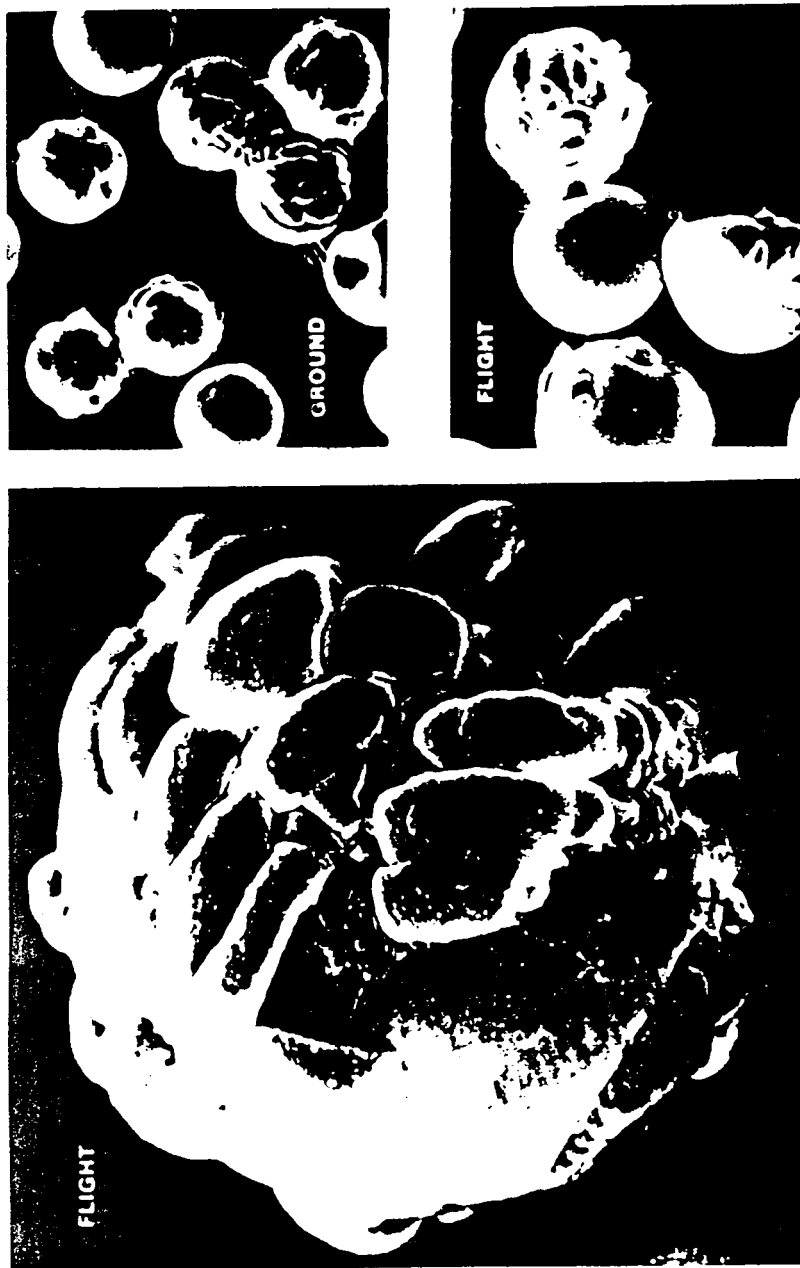
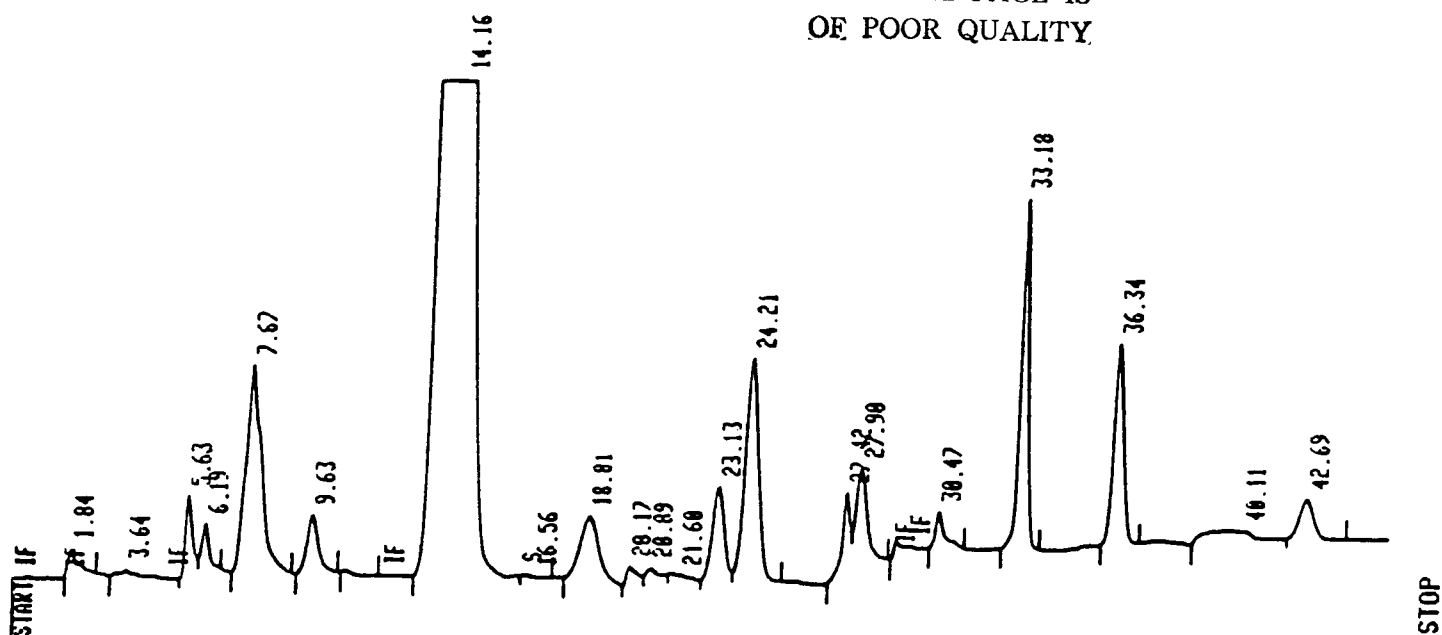


Figure 8-5.- Fixed 24.5 hours after mixing cells and beads.

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ESTD

RT	AREA	TYPE	CAL#	AMOUNT
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3.64	130510	BP		0.176
5.63	622690	PV		0.841
6.19	415010	VB	1	0.481
7.67	3570400	PB	2	4.420
9.63	723880	PB	5	0.716
14.16	2.5584E+08	SBB	7R	277.780
18.81	1462200	BP	10	1.665
20.17	238530	PV		0.322
20.89	251670	VV		0.340
21.60	299460	VV	11	0.324
23.13	1363600	VV	12R	1.606
24.21	3478200	VB	13	4.049
27.42	723190	BV	14	0.970
27.90	1358600	VB	15	1.854
30.47	378250	BB	16	0.425
33.18	2998800	PB	18	2.926
36.34	2213800	PB	19	3.776
40.11	707250	BV		0.955
42.69	748720	VB	21	1.017

TOTAL AREA= 2.7777E+08
MUL FACTOR= 1.0000E+00

Figure 8-6.- Amino acids analysis (HPLC) of serum-free medium
(no cells present).